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Progesterone and estrogen are the key steroidal hormones involved in breast development and tumorigenesis. The effects of progesterone and estrogen are mediated through specific intracellular receptors and the status of these receptors in breast tumors has been used as an important prognostic indicator of probability of disease free survival and response to hormonal therapies. The progesterone receptor (PR) is composed of two isoforms, PRA and PRB which have different transactivation functions *in vitro* and are likely to have different physiological roles in breast development and tumorigenesis. To date no *in vivo* model exists to address this question. To generate such a model, we have proposed to selectively ablate expression of PRA or B in mice by gene targeting. We have devised two alternative strategies to introduce subtle mutations into the mouse PR gene by gene targeting in embryonic stem (ES)cells. To date, we have produced three out of four gene targeting constructs needed for homologous recombination using these strategies. Further, we have successfully completed the first of two targeted integration steps in ES cells using one strategy. Finally, using the PR null mutant mice we previously generated, we provide evidence that the cell cycle protein D1 cyclin may be a key mediator of the mammary developmental responses that are specific to progesterone.

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Orla M. Connelly Oct 2 '96

PI - Signature Date

(4) Table of Contents

INTRODUCTION

Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (1-3). In the case of mammary gland tumorigenesis, the effects of progesterone and estrogen on carcinogenesis can be both stimulatory and inhibitory and are dose and stage dependent (4). These hormonal effects are mediated by specific high affinity intracellular receptor proteins that are members of a superfamily of related transcription factors (5,6). Binding of steroids to these receptors results in the formation of activated receptor dimers that bind to specific enhancer DNA elements located in the promoter regions of hormone-responsive genes (7,8). The activation or repression of these genes represents the manifestation of the hormonal response.

The mammary gland is the site of milk production and secretion and, in females, is a major site of tumorigenesis (9). Mammary gland development occurs during the fetal, post-natal and adult stages of life (10). The development of the mammary gland occurs primarily post-natally and is directed by a complex signal transduction interplay between hormonal (polypeptide and steroid) and growth factor signals. During pregnancy, progesterone and estrogen promote growth and differentiation of normal mammary tissue by regulating ductal branching, alveolar formation (11) and lobuloalveolar development (12). Studies on the ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones have indicated that receptors for estrogen and progesterone (ER and PR respectively) are present in both stromal and epithelial cells, and begin to exert effects on terminal end bud proliferation at 4 and 7 weeks of age, respectively (13). Furthermore, it now appears that epithelial cells, which can express receptors for estrogen and progesterone, are the major sites of primary mammary carcinomas (14).

Although the general consensus on progestin action in the uterus is that progesterone inhibits the proliferative effect of estrogen and acts as a differentiating hormone, this concept cannot be extended to the breast (3). Considerable evidence has accumulated to implicate progesterone in the proliferation of normal mammary epithelium in virgin animals (15) and in the development of the lobular-alveolar structure in mammary glands of pregnant animals (16). Unlike estrogen action, progesterone is a mitogen, not only in the epithelium of the terminal end buds, but also in the ductal epithelium (17). Depending on the time of administration and the dosage used, progestin agonists have been shown to reverse the anti-tumor effects of the anti-estrogen, tamoxifen, and induce tumor growth (18). The observation that the tumor inhibitory effect of tamoxifen can be reversed by progestin agonists (18) together with the stage and dose dependent carcinogenic activity of progestin agonists (3) suggest that some of the effects of ERs may be mediated by PRs whose expression is known to be induced by estrogen (19). Taken collectively, the above data supports the proliferative effect of progesterone in normal breast development and in contributing to the oncogenic potential of the breast. Conversely, studies using the carcinogen-induced rat mammary tumor model (20) have shown that early pregnancy (21) or the administration of high doses of progesterone and 17β estradiol (22) shortly after the onset of sexual maturity were effective in reducing the susceptibility of the mammary gland to chemical carcinogenesis. Thus, progesterone appears to have both stimulatory and inhibitory effects on mammary gland tumorigenesis that are stage and dose dependent.

From a clinical standpoint, the estrogen and progesterone receptor status of breast tumors is an important prognostic factor in determining the probability of disease free survival and

response to hormonal therapy (2,23). Breast tumors that contain functional ERs and PRs have a higher response to hormonal therapy and higher disease free survival probability (2). However, as tumorigenesis progresses, the disease develops to a state that is characterized by a lack of ERs and PRs and a resistance toward hormonal and cytotoxic therapies.

It has been established that PR is composed of two hormone binding forms *in vivo*, termed PR_A and PR_B (24,25). It is thought that the A and B forms arise as a result of either alternate initiation of translation from a single mRNA (26) or by alternate transcription from promoters within the same gene (27). These receptor isoforms differ only in that PR_B contains an additional stretch of amino acids at the amino terminus of the receptor. Previous experiments have shown that these proteins exhibit different promoter specificities for target gene activation (28) while binding to the same enhancer DNA element (29). Remarkably, recent data have implicated a novel repressor function as well as an activator role for PR_A (30). Depending on the promoter and cell context, PR_A was shown to act as a potent transdominant repressor of PR_B-mediated gene transcription. In addition, the repressor function of PR_A was found to influence the activity of other members of this superfamily of transcription factors which included the glucocorticoid, mineralcorticoid and androgen receptors. Intriguingly, recent transient cotransfection experiments have revealed that PR_B, when occupied by progestin antagonists, can activate transcription (31). Furthermore, this unusual PR_B mediated antagonist transactivation can be dominantly inhibited by the PR_A isoform. This apparent paradoxical stimulatory action of progesterone antagonists via PR_B, if substantiated, would prompt a reevaluation or the potential efficacy of any chemoprevention strategy involving these 'anti-progestins' in the treatment of breast and uterine cancer.

Although, for two decades, the PR has been shown to be composed of two receptor isoforms, the specific physiological role for each of these two PR subtypes in normal breast development, tumor initiation and progression, has yet to be established. However, the existence of both these receptors in different species and tissues, and the elaborate mechanisms regulating their expression suggests that the absolute and relative levels (receptor status) of PR_A and PR_B in a progestin target cell are critical for the correct cellular response to progesterone and its antagonists. The equimolar expression of both forms of the PR in the same cell would allow the possible formation of two homodimers and one heterodimer (A:A, B:B and A:B). The potential existence of three dimeric forms of PR, each having different transcriptional regulatory specificities, would serve to further expand the repertoire of physiological responses to progesterone. Although breast tissue may contain an overall equimolar ratio of PR_A to PR_B, it is quite possible that different cell types of this tissue, for example epithelial and stromal cells, may have a different ratio which is critical for the normal functioning of these cells. Therefore alterations in the ratio of PR_A to PR_B, would be expected to contribute to an altered susceptibility of these cells to carcinogenesis and have a dramatic effect on the cellular response to progesterone agonists, antagonists, other steroids and growth factors and proto-oncogenes regulated by progesterone.

An additional level of complexity in the involvement of these receptor isoforms in mammary gland development and tumorigenesis arises from influence of growth factors and proto-oncogenes such as epidermal growth factor (EGF), c-myc and cyclin D1 which have been shown to be increased by progestins in cultured human breast cancer cell lines (32). These mitogens may represent "early target" genes for progesterone which may act via autocrine and

paracrine mechanisms to influence breast tissue proliferation and differentiation. At this stage, it is not known which of these gene products are modulated by either one or both isotypes of PR.

Purpose of the Present Work

Based on the above observation, we have proposed the following hypothesis: During breast development and tumorigenesis, progesterone mediates its mitogenic effect through two receptor isoforms, PR_A and PR_B. We predict that, *in vivo*, PR_A and PR_B have distinct physiological effects and that the ratio of PR_A to PR_B is a key determining factor for normal breast development, oncogenic potential and carcinogenesis.

We have used a genetic approach to test the above hypothesis. Two fundamental questions regarding the role of progesterone and its receptor in breast development are being addressed. These are: (1) What is the *in vivo* functional significance of progesterone in general breast development? and (2) What is the *in vivo* functional relevance of the A and B forms of PR in normal breast development and tumorigenesis. These questions will be answered by the physiological analysis of mutant mice deficient in both forms of the receptor (PR_{A+B}-ve) and mouse lines deficient in either the A or B form of the receptor (PR_A-ve and PR_B-ve respectively). The generation of these mouse models will be accomplished by the mutation of the endogenous mouse PR gene by homologous recombination (gene targeting) in mouse embryonic stem (ES) cells. Pluripotent ES cells carrying the mutated PR allele will be injected into mouse blastocysts where they will become the progenitor cells of most of the embryonic tissues including the germ line. Germ line transmission of the mutated PR allele will allow the creation of mouse strains that are heterozygous and homozygous for the mutant PR gene.

PROGRESS.

During the past year we have focused our efforts in two areas: 1) To selectively mutate the PR A or B genes in embryonic stem cells; and 2) To identify PR dependent target genes that mediate its proliferative and differentiative effects in the mammary gland and whose regulation is disrupted in PR-/- homozygous null mutant mice.

1) SELECTIVE ABLATION OF PR A OR B PROTEINS IN EMBRYONIC STEM CELLS.

To define significance of A or B isoforms of progesterone receptor (PR) *in vivo* we have proposed to introduce mutations into the mouse germ line, and generate the mutant mouse models that will then express either A or B or PR. Our approach to create mouse models, which will express only A form of PR is to mutate the initiating ATG for B (ATG_B) PR. Therefore, the open reading frame (ORF) for PR B form will be destroyed. Similarly, mutation of ATG site for PR A (ATG_A) will create mouse model that produces only B form of the receptor.

Although over 200 genes have now been ablated in mice through gene targeting approaches, none to date have been successfully altered in mice by introducing point mutations into functional genes using these approaches primarily because selective markers used to select for uptake of targeting vectors must be removed in a relatively inefficient two-step process before

generating chimeric mice. Further, the two step procedures required for this targeting event can compromise the viability of embryonic stem cells before microinjection into mice. Thus, our objective to introduce these subtle mutations into PR gene represents a technical challenge that is more complex than our previous null mutation of the PR gene. We therefore have adopted two parallel technical approaches to accomplish this goal. The first involves the double replacement strategy also referred to as the "tag and exchange approach". With this strategy (Fig. 1) the first targeting step uses conventional gene targeting with positive selection to introduce positively (neo resistance gene) and negatively (thymidine kinase gene) selectable marker sequences (Fig. 1A) into the target gene (Fig. 1B) and tag the allele (Fig. 1C) of interest. In the second step, sequences homologous to the tagged target gene carrying a desired subtle mutation (Fig. 2D) are used to replace (exchange step) the marked allele (Fib. 1E). To ensure that both markers are efficiently expressed, we used 4kb of tagging sequences from the plasmid, pPFKneoNTRtkpA that contain at 5' end 0.5 kb the mouse phosphoglycerate kinase 1 gene (Pgk-1) promoter, followed with 0.8 kb of bacterial neomycin phosphotransferase (neo) cassette, 0.58 kb fragment of 5' nontranslated region (NTR) of the encephalomyocarditis virus, and finally, a 2.1 kb 3' end fragment which includes the thymidine kinase (tk) gene and polyadenylation signal sequences from tk and Pgk-1 genes. The tagging sequences were then flanked with 1.2kb encoding part of exon 1of PR genomic DNA on the 5' side and a 5.5kb fragment encoding parts of exons 1 and 2 on the 3' side. The linearized vector was electroporated into embryonic stem cells and the cells were selected for neomycin resistance using G418. The G418 resistant clones were screened for homologous recombination events using Southern analysis on genomic DNA isolated from actively growing ES cells. Genomic DNA was digested with Hind III, and blots after electrophoresis were probed with radioactive 0.5 kb SacI-EcoR1 PR genomic fragment located 5' to the PR sequences used in the targeting vector. The wild type PR gene is represented by a 5.5kb HindIII band while introduction of the selectable markers into the targeted PR allele provides an additional Hind III restriction site resulting in a second hybridizing band of 2.0kb. An example of the results of this screening is shown in Figure 2. Employing this screening strategy, we achieved a targeting frequency of 15% at the PR locus. Thus, we have successfully accomplished the first step of this approach and tagged the PR locus so that the A or B mutant genomic fragments can be exchanged for the selectable markers in the second step.

Preparation of A and B targeting DNAs for second targeting step.

In order to selectively express the PR B protein, we have mutated the ATG start site for the PR A protein using Quick Change Site-Directed Mutagenesis kit (Stratagene). We are in the process of mutating the ATG translation initiation site for the B protein in order to selectively express PR A.

Mutation of ATG_A triplet (1.5 pmA)

The sequence of wild-type and mutated region is shown in Figure 3. The ATG triplet which initiates translation of PR A was replaced with GCT triplet resulting in a single conservative amino acid exchange of Met for Ala. An additional single base change at third position of triplet encoding Ser 167 was also introduced in order to generate novel NheI site for screening purposes. The mutations were confirmed by sequencing of both DNA strands.

A. (163) Ser Pro Leu Met Ser Arg Pro
TCC CCG CTC ATG AGT CGG CCA

B. (163) Ser Pro Leu Ala Ser Arg Pro
TCC CCG CTC CTG AGC CGG CCA
NheI

Figure 3. Comparison of wild-type (A) and mutant sequences (B) in the region containing the translation start codon for PR_A.

In summary, we are at the final stage for selective mutation of PR A in ES cells. We will electroporate this targeting vector into ES cells and select for its ability to replace the PR tagging vector by growth of the cells on FIAU containing media to select for removal of the TK marker gene. Because the FIAU selection step relies on a second and inefficient round of homologous recombination in ES cells, we also have adopted a second independent approach described below to introduce the PR mutations.

SELECTIVE MUTATION OF THE A AND B TRANSLATION SITES USING TAG AND CRE-LOX P MEDIATED RECOMBINATION.

The CRE-*lox* P approach is a two step mutation strategy that uses a modified PR targeting or tagging vector in the first step in which the neo and tk selectable markers are flanked by two lox-P DNA sequences which are specifically recognized by the site specific recombinase, CRE recombinase. The more recent approach does not require a second step of homologous recombination but exploits the ability of CRE recombinase to catalyse site specific recombination between the two lox-P sites and delete the selectable markers in between. Thus, in the first step (Fig. 4), the PR tagging vector is modified to introduce the selectable markers flanked by lox-P sites on either end into intron 2 of the PR gene so that the lox-P sites are flanked by 3kb of PR genomic sequence encoding exons1-2 **including the A or B initiation site mutations** at the 5' end and 4kb of PR genomic sequence encoding the remainder of intron 2 and the 3'end. To date we have successfully constructed the targeting vector for mutation of the A start site and are ready for electroporation of this vector into ES cells. We have also obtained the CRE-recombinase vector for transient transfection into ES cells at the second step and site specific recombination.

2) IDENTIFICATION OF TARGET GENES THAT MEDIATE PR DEPENDENT PROLIFERATION AND DIFFERENTIATION.

Our second objective was to begin to identify biochemical targets of the progesterone receptor that may mediate its growth and differentiative effects in the mammary gland and whose deregulation may contribute to tumorigenesis in the tissue. In the original grant application, we targeted several growth factors and cell cycle proteins for analysis that had been shown by other groups to be regulated by progesterone in breast cancer cell lines. In the first phase of these analysis we have focused on a comparative analysis of the estrogen and progesterone regulation of the D cyclins, and in particular D1 cyclin for the following reasons: 1) D1 cyclin is induced by

mitogenic stimuli during the G1 phase of the cell cycle and mediates their proliferative effects by activating cyclin dependent kinases to remove the cell cycle block at the G1/S phase checkpoint and allow progression through mitosis; 2) D1 cyclin is induced by progesterone in the G1 phase in T47D breast cancer cells resulting in accelerated cell cycle progression through G1 in these cells; 3) Both D1 induction and cell cycle progression are blocked by progesterone antagonists in these cells; 4) overexpression of D1 in the mammary gland of transgenic mice results in mammary adenocarcinomas and the gene is localized on human chromosome 11q13 in a region that is amplified in 15-20% of mammary carcinomas. Finally and most strikingly, the mammary phenotype of our PR-/- null mutant mice shows significant overlap with that of the D1 cyclin null mutant mice and strongly supports a D1 cyclin mediated proliferative and differentiative response to progesterone. To determine whether D1 cyclin can selectively mediate the effects of progesterone (P) in the mammary gland, we compared the temporal expression patterns of D cyclin mRNA transcripts in wild-type versus PR-/- null mutant mice after treatment with estrogen (E) alone or E and P. The results of these experiments are shown in figure 5, panels A and B. Panel A compares the expression of D1 and D2 cyclins and GAPDH controls in the mammary glands of wild-type mice treated with E alone or E and P for 1, 4, 8, 11 and 15 days. In contrast to D2 cyclin, there appears to be a significant induction of D1 cyclin in animals that were cotreated with E + P relative to E alone that is most obvious after 11 and 15 days of treatment. When we carried out similar analysis of the PR-/- mice, however, we observed no P dependent induction of D2 mRNA as expected but the D1 induction in E and P treated mice is now lost (panel B). These data are consistent with the hypothesis that D1 cyclin is a selective target for PR in mammary cells. However, to confirm that this P dependent induction of D1 is not simply a function of P induced epithelial expansion in the gland, we are currently analyzing the D1 protein responses under these conditions at the single cell level by immunohistochemistry. Further, we will determine whether these responses are specific to the mammary gland by comparison with uterine cyclin response to these hormones. If these responses prove to be mammary specific, then D1 cyclin may be a primary hormone responsive target whose deregulation mediates mammary specific tumorigenesis.

CONCLUSIONS

During the past year we have made progress in the development of two alternative strategies to ensure efficient selective mutagenesis and ablation of expression of the PR A or B proteins. We expect to have A or B null mutant mice within the next 6-8 months and begin analysis of their selective physiological roles. Using the PR null mutant mice we have already generated we have identified D1 cyclin as a potentially important cell cycle regulatory target that appears to be preferentially induced by progesterone in the mammary gland and may be a key mediator of the normal proliferative and/or differentiative effects of the hormone. Our priorities for the next year have not changed relative to the original proposal. We expect to successfully produce A or B null mutant mice using either of the embryonic stem cell targeting strategies we have developed and will begin analysis of their mammary phenotype and hormonal responses.

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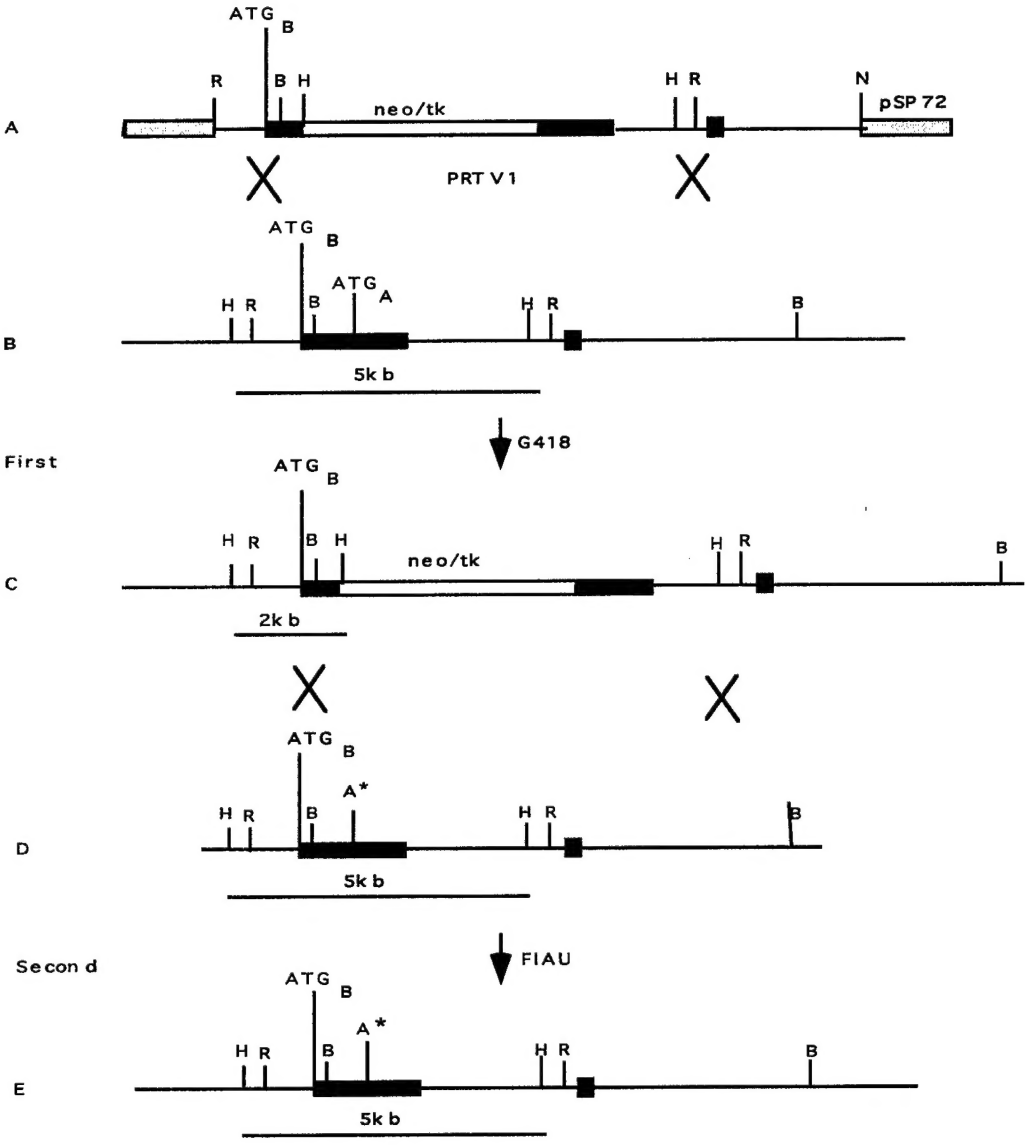


Figure 1. A scheme of the double replacement procedure:
An example for PR deficient mutant. B, Bam HI; R, Eco RI; H, HindIII.

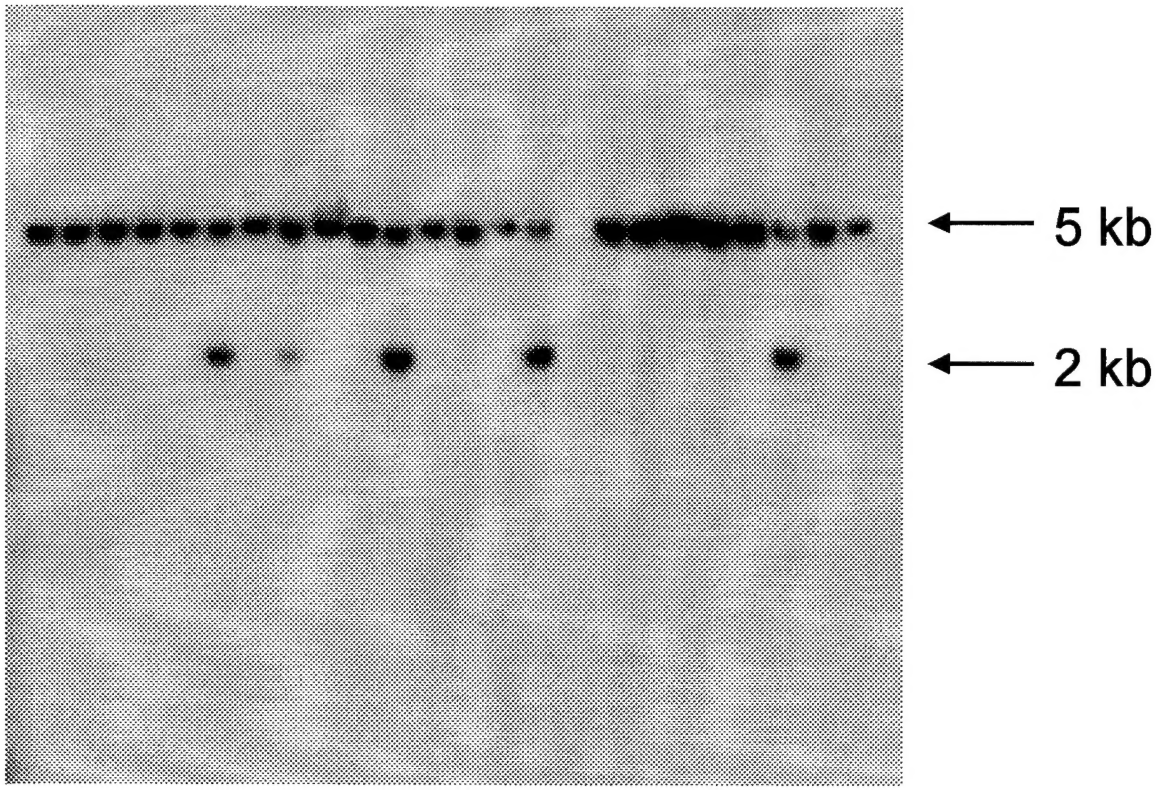
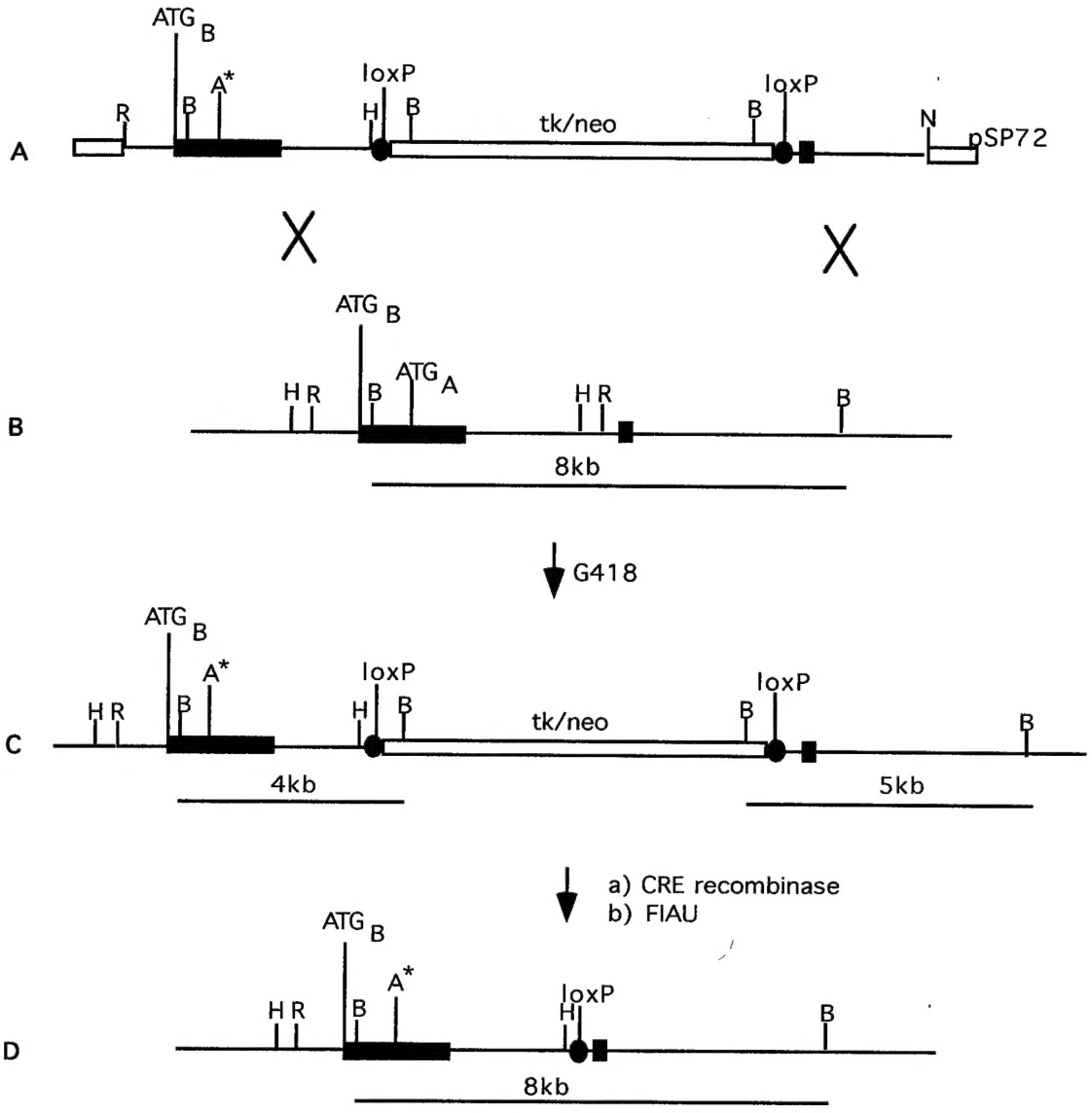
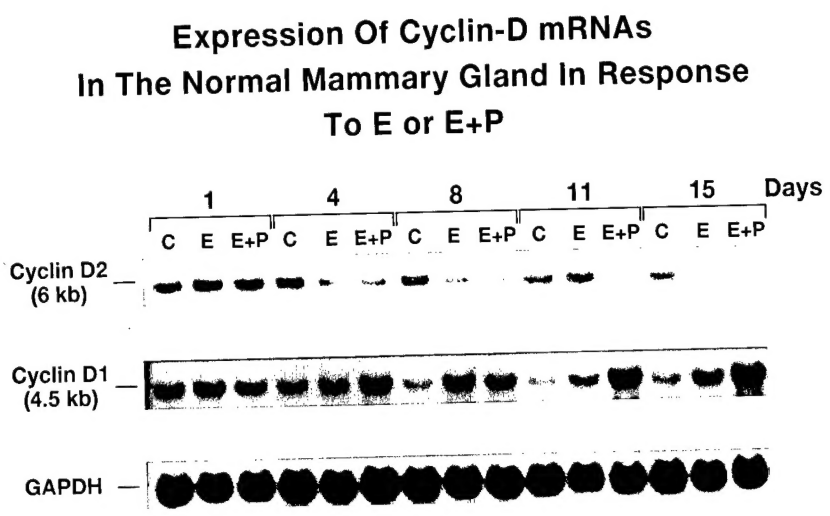


Figure 2. The Southern blot analysis of ES cells



Figures 4. A scheme of the CRE-lox P mediated strategy: An example for PRA deficient mutent. B, Bam HI; R, Eco RI; H, HindIII; N, NotI.

Panel A



Panel B

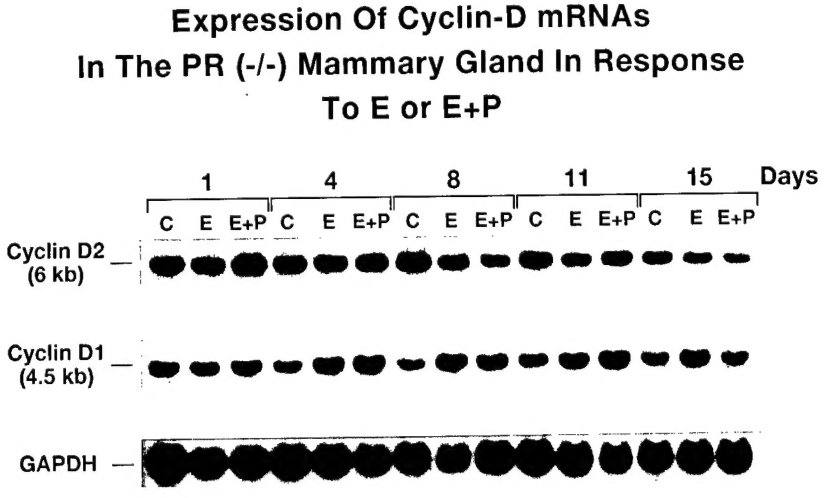


Figure 5. Northern analysis of D cyclin mRNA expression in normal (panelA) and PR-/- mammary glands from untreated mice (C), mice treated with estrogen alone (E) or with estrogen plus progesterone (E+P) for the indicated periods in days.